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P-glycoprotein and Topoisomerase II α Expression in Advanced Gastric Cancer Patients: Association with Clinicopathological Findings

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Abstract: Comparative study of markers of drug resistance in cancer tissues may be extremely helpful in selection of effective chemotherapeutic regimen. P-glycoprotein (P-gp) and Topoisomerase II α (Topo II α) are two fundamental proteins in multi-drug resistance phenomenon (MDR). This study determined the expression and significance of P-gp and Topo II α proteins in advanced gastric carcinomas and correlated molecular alterations with clinicopathological findings. Tissue samples of 35 patients with advanced type gastric adenocarcinoma were analyzed. Immunohistochemical techniques were applied using primary antibodies for P-gp or Topo II α and LSAB2 detection kit (Dako-Denmark). Positive immunostaining for P-gp and Topo II α were observed in 42.9% and 17.2% of tumor samples, respectively. Negative expressions of P-gp and TopoII were associated with sex (p = 0.035 and 0.0001) and histological grade (p = 0.021 and 0.0001), respectively. Unlike P-gp, an inverse relationship between Topo II α expression and size of tumors (p = 0.012) and lymphatic invasion (p = 0.013) were observed. Considering the key role of positive P-gp or negative Topo II α expression in MDR, it could be concluded that groups of patients with P-gp over expression (42.9%) or lack of Topo II α expression (82.8%) would less likely benefit from available chemotherapeutic regimens. Therefore, we highly recommend determination of tumor status for expression of tumor markers such as P-gp and Topo II α, before deciding about effective chemotherapeutic regimen for patients with gastric cancer.

Key words: Gastric carcinoma, immunohistochemistry, MDR, P-gp, Topo II α

INTRODUCTION

Gastric carcinoma usually shows little or no response to chemotherapy which thus makes difficult in advance cases (Lim *et al*., 2005). Resistance to antineoplastic drugs is one of the major reasons of failure in chemotherapy-based treatment of malignant tumors (Katoh, 2004). P-glycoprotein (P-gp) is an ATP dependent membrane protein and transports a wide range of cytotoxic drugs out of tumor cells. The tumor cells which expressed P-gp have a decreased accumulation of cytotoxic substrates and thus are resistant. The classical MDR phenotype of human malignancies is mediated by drug extrusion by the adenosine triphosphate binding cassette (ABC)-transporter P-glycoprotein (MDR1/P-gp) (Stege *et al*., 2004).

Topo II α separates chromosomes at the end of mitosis and is also the target for various chemotherapeutic agents. Expression of this enzyme has been demonstrated to increase rapidly at the end of the S to G2/M phase and decrease after the completion of mitosis, therefore it is suggested that Topo II α immunostaining can detect proliferating cells in routinely processed tissue sections and can indicate the altered Topo II α expression in human cancers, which may be related to the sensitivity of Topo II α targeted chemotherapeutic agents (Yabuki *et al*., 1996).

As these proteins are common targets for several usual cytotoxic drugs including Doxorubicin (Stege *et al*., 2004; Yabuki *et al*., 1996), therefore this study was designed to prove the protein levels of P-gp and Topo II α in advanced gastric tumors as well as their clinicopathological significance, significant immunophenotypes and finally the clinicopatholigcal significance of each immunophenotype for the first time to use them together as prognostic or predictive markers in related further studies.

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MATERIALS AND METHODS

Patients and sample characteristics: Samples of 35 advanced gastric adenocarcinoma cases were randomly obtained from patients who underwent surgery at two different university hospitals (Shohadaye Tajrish and Imam Khomeini) during the year 2000-2003. The patients included were Iranian from different geographical locations with Iran. Based on the designed questionnaire, data were collected for age at surgery, sex and pathological diagnosis. Histopathological data contained tumor pathological type, tumor size, histological differentiation (malignancy grade), stage and lymphatic invasion.

P-gp and Topo II α immunohistochemical analyses: As previously described by Arbabi *et al*., (2005) dewaxed and rehydrated tissue sections were subjected to antigen retrieval using microwave oven and boiling citrate buffer (pH = 6.0). Endogenous peroxidase activity and nonspecific binding sites were blocked by incubating sections in 3% hydrogen peroxide in methanol for 30 min and 5% BSA for 60 min, respectively. Sections were then incubated overnight at 4°C with P-glycoprotein mouse monoclonal antibody (clone c-494 Dakocytomation) that recognizes the membranous expression of the human P-glycoprotein and Topo II α mouse monoclonal antibody (Clone Ki-S1 Dakocytomation) that recognizes the nuclear expression of human Topo II α protein. The primary antibodies were used at dilution of 1:200 and 1:100, respectively. The results were visualized using the Streptavidine-biotin immunoperoxidase detection kit (LSAB2; Dakocytomation -Denmark) and DAB chromogen (Dakocytomation-Denmark) based on the manufacturer's instruction with necessary modifications. Sections were also counter stained with Meyer's haematoxyline. In each series, a section in which incubation with the primary antibody was omitted used as negative control. The ideal staining conditions were established in our preliminary experiments. Staining was considered negative only after careful examination of the entire tissue section. Semiquantization of the intensity and number of positive tumor cells was performed by two independent pathologists (B. M. and M. D.) blinded to the clinical outcome. In cases in which the observers disagreed, the immunohistochemical scoring was repeated to agree on same scoring by both observers. Tumor samples were then classified into four categories based on the membranous or nuclear expressions of markers. Based on quantization of the intensity and number of positive tumor cells, tumor cells were scored as 3+ if they had strong staining (>50%), 2+ if they had moderate staining (25-50%), 1+ if they had mild staining (5-25%) and 0 if staining was <5% or no staining. According to the guidelines for pathological studies on gastric carcinoma histological grade and pathological stage of tumors were also determined (Rubin and Farber, 1998).

Statistical analyses: For the statistical analyses, descriptive data were expressed as the mean±SD. To compare means of continuous variables, independent sample t-test, to compare discontinuous variables Man Whitney u-test and to determine the correlation between molecular features and clinicopathological parameters the spearman's correlation test were performed using SPSS12 software (Robert *et al*., 2000). The correlation between scores of marker expression and tumor size, histological differentiation, lymphatic metastasis, as well as patients' sex and age at surgery were statistically evaluated.

RESULTS

The results of clinicopathological features of 35 evaluated gastric cancer patients showed that there was no significant difference between the mean ages of men (61.7±11.118) and women (63.1±11.901) (p = 0.756) (Table 1).

Status of Topoisomerase II expression in gastric carcinoma samples: As showed in Table 2, out of 35 evaluated patients 6 (17.2%) showed positive expression of this marker and most of them 29 (82.9%) didn't show any expression according to our scoring method. In the group of positive expressions no strong staining was observed and positive cases were equally divided to 3 (50%) mild and 3 (50%) moderate staining. Figure 1(a-c) show moderate and weak Nuclear Topo II α expression in moderately differentiated advanced gastric adenocarcinoma, respectively.

Table 1: Clinicopathological features of gastric cancer patients

Age of patients	61.87±10.6997
Male	61.7+11.118
Female	63.1+11.901
Sex:	
Male	24 (68.6%)
Female	11 (31.4%)
Tumor size:	5.02±2.8223 (Range 0.8-10.5 cm)
Lymphatic invasion:	
Positive	17 (48.7%)
Negative	18 (51.3%)
Histological Grade	
Well differentiated	11 (33.3%)
Moderately differentiated	12 (36.4%)
Poorly differentiated	7 (21.2%)
Undifferentiated	3 (9.1%)
Stage	
Early	0 (0%)
Advanced	35(100%)

Table 2: Status of marker expressions in gastric carcinoma patients

Markers	Positive	Negative	1+	2+	3+
P-gp	15 (42.9%)	20 (57.1%)	11 (31.4%)	3 (8.6%)	1 (2.9%)
Topo II α	6 (17.2%)	29 (82.9%)	3 (8.6%)	3 (8.6%)	0

Status of P-glycoprotein expression in gastric carcinoma samples: Out of 35 evaluated patients 15 (42.9%) of patients showed positive expression of this marker and the other ones 20 (57.1%) didn't showed any expression according to our scoring method (Table 2). In the group of positive expressions, 11 cases (73.3%) showed mild staining, 3 cases (20%) moderate staining and only 1 case (6.7%) strong staining. Figure 1d and show weak and moderately membranous expression of P-gp in moderately differentiated advanced gastric adenocarcinoma respectively. Figure 1f shows lack of marker expression in moderately differentiated advanced gastric adenocarcinoma (Negative control).

Clinicopathological significance of P-glycoprotein: The results of present study indicate significant relationship between positive expression of P-gp and gender ($p = 0.035$) as well as histological grade ($p = 0.001$). Other clinicopathological factors showed no association with P-gp expression (Table 3).

Clinicopathological significance of Topoisomerase II α: As well as P-gp, Topo II α negative immunostaining was also significantly related to gender ($p = 0.0001$) and histological grade ($p = 0.0001$). Three additive clinicopathological factors showed significant relationships with the lack of Topoisomerase II expression. These factors were lymphatic invasion ($p = 0.013$) and tumor size ($p = 0.012$) and age ($p = 0.004$) as described in Table 3.

Correlation between P-gp and Topo II α expression: According to the values mentioned in Table 2, four different expression patterns of markers were identified among the cases (data not showed). Based of these expression patterns of markers, tumors were classified to four immunophenotypes: a) P-gp+/Topo II+ (3/35, 8.57%), b) $P\text{-gp}^{+}$/Topo II^{-} (12/35; 34.3%), c) $P\text{-gp}^{-}$/Topo II^{+} (3/35; 8.57%) and d) $P\text{-gp}^{-}$/Topo II^{-} (17/35; 48.57%). The prevalence of $P\text{-gp}^{-}$ /Topo II^{-} (48.57%) immunophenotype was significantly higher than other immunophenotypes in evaluated gastric carcinoma patients ($p = 0.035$).

Correlation between age and P-glycoprotein, Topo II α expression in group of females: The mean age of females with positive P-gp expression (55.5±14.24) was significantly ($p = 0.05$) lower than females with P-gp

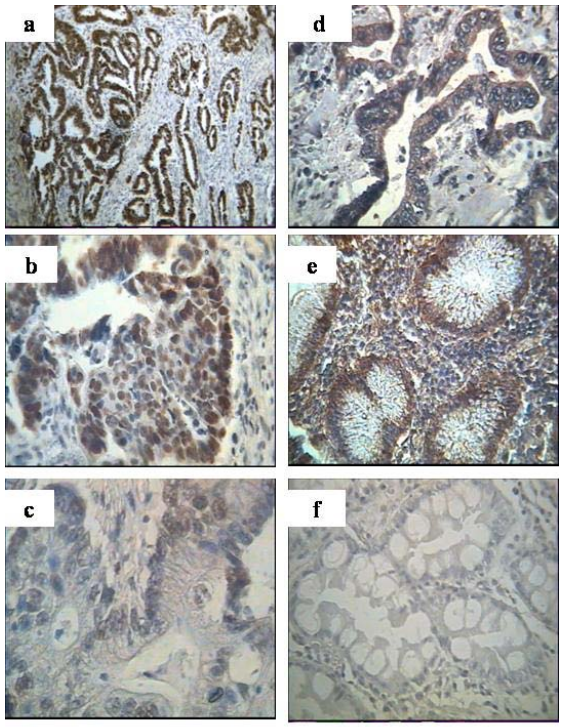

Fig. 1: Immunohistochemical staining of P-gp and Topo II a in tumor samples of patients with advanced gastric cancer. Tumor sections were stained using primary monoclonal antibodies for P-gp and Topo II a as described in materials and methods. a) Moderate Nuclear Topo II α expression in moderately differentiated advanced gastric adenocarcinoma (x100), b) Moderate Nuclear Topo II α expression in moderately differentiated advanced gastric adenocarcinoma (x400). c) Mild Nuclear Topo II α expression in moderately differentiated advanced gastric adenocarcinoma (x400). d) Moderately membranous expression of P-gp in moderately differentiated gastric adenocarcinoma. e) Strong membranous expression of P-gp in moderately differentiated gastric adenocarcinoma f) Negative expression of markers in moderately differentiated gastric adenocarcinoma (Negative control)

negative expression (70.67±7.5). The same difference was not observed between females with Topo II α positive or negative expression (Table 4).

Correlation between age and P-glycoprotein, Topo II α expression in group of males: The mean ages of males with positive P-gp and /or Topo II α expression were not significantly differed from negative ones (Table 4).

Table 3: Clinicopathological significance of P-gp and Topo II α expression in gastric carcinoma

Variable	No. of patients	P-gp(+) n = 15	P-gp (-) n = 20	p-value	Topo II (+)n = 6	Topo II α (-) n = 29	p-value
Age							
Over 62	19	10	9	0.424	3	16	0.004**
Under 62	16	5	11		3	13	
Tumor size							
Over 5 cm	18	6	12	0.664	2	16	0.012*
Under 5 cm	17	9	8		4	13	
Histological grade							
G1[1]	23	10	13	0.021*	4	19	0.0001***
G2[2]	12	3	7		2	10	
Lymphatic invasion							
Positive	17	8	9	0.804	3	14	0.013*
Negative	18	7	11		3	15	

Note 1: G1 means lower malignancy grade which contains well differentiated and moderately differentiated tumors, 2: G2 means higher malignancy grade which contains poorly differentiated and undifferentiated tumors

Table 4: Comparison of mean age and P-glycoprotein, Topo II α expressions in males and females

Groups	P-gp(+)	P-gp(-)	p-value	Topo II α(+)	Topo II α(-)	p-value
Mean age of males	64.3±11.7	59.3±10.40	0.112	60.8±14.9064	61.95±10.44	0.841
Mean age of females	55.5±14.24	70.67±7.5	0.05*	52	62.7±10.65	0.365

Table 5: The clinical value of gender on the expression patterns of P-gp, Topo II α, P-gp$^+$/Topo II α$^-$ and P-gp$^-$/Topo II α$^-$ immunophenotypes

Markers	Male	Female	p-Value
P-gp (+)	12	3	0.035*
P-gp (+)	12	8	
Topo II α(+)	5	1	0.0001***
Topo II α(+)	19	10	
P-gp$^+$/Topo II α$^-$	9	3	0.008**
Other Immunophenotypes	15	8	
P-gp/Topo II α$^-$	11	6	0.167
Other Immunophenotypes	13	5	

Correlation between P-gp$^-$/Topo$^-$ immunophenotype and clinicopathological findings: This immunophenotype was not significantly correlated with clinicopathological data. The same results were observed in P-gp$^+$/Topo II$^+$ and P-gp$^-$/Topo II$^+$ cases (data were not showed).

Correlation between P-gp$^+$ / Topo$^-$ immunophenotype and clinicopathological findings: Current results indicate significant relationship between P-gp$^+$/Topo$^-$ immunophenotype and gender of patients (p = 0.005). Table 5 shows the clinical value of gender on the expression patterns of P-gp, Topo II α as well as P-gp$^-$/Topo II α$^-$ and P-gp$^+$/Topo II α$^-$ immunophenotypes.

DISCUSSION

Due to the clinical values of P-gp and Topo II a in cancer chemotherapy and lack of comprehensive study on the clinical significance of these markers in advanced gastric carcinoma, the status of P-gp and Topo II a expression and their clinicopathological significance were analyzed. There is also no data on P-gp and Topo II α expression in Iranian patients with gastric carcinoma. To our knowledge, this is also the first study to characterize the clinicopathological significance of P-gp/Topo II α in advanced gastric adenocarcinoma. We determined the protein expression of these two important proteins in 35 patients who are all in advance stages of aggressive adenocarcinoma. In present study 15 of 35(42.9%) cases showed P-gp expression but in previous studies, positivity rates of 10% to 80% have been reported (Fan *et al*., 2000; Fuji *et al*., 1995; Mizokuchi and Yamada, 1990; Dhar *et al*., 1995; Fuji *et al*., 1994; Ramesh *et al*., 2003). Although this wide difference may be related to geographical factors, due to technical differences, sensitivity rates of various molecular techniques used, or the clone of primary antibodies but we suggest the possible role of gender in this area.

We found that P-gp expression had a significant relationship with gender of patients (p = 0.035).That means despite the equal number of male patients who had positive (n = 12) or negative (n = 12) expression of P-gp, female negative ones (n = 8) were 2.5 folds more than positive cases(n = 3).Furthermore despite males, the mean age of positive females was significantly lower than negative cases (p = 0.05).

Our findings is comparable with those of other investigators who have demonstrated the presence of approximately 2.4-fold higher P-gp levels in men compared with women in normal liver and hepatic neoplasm (Schuetz *et al*., 1995).This finding suggests that there is maybe an association between sex-steroid hormones and P-gp expression which are substrates for P-gp–mediated transport and are also able to induce P-gp expression at both the protein and mRNA level *in vitro* (Kim and Benet, 2004). Stimulation of P-gp ATPase catalytic activity by steroid hormones is also observed, suggesting physical interactions and identifying a need for further investigations to understand the *in vivo* effects of endogenous and synthetic steroid

hormones on the expression and function of P-gp (Kim and Benet, 2004).

Although the possible effects of steroid receptors on the expression of P-gp and chemotherapy outcome could be concluded from this preliminary *in vivo* study but the evaluation of the coexpression of P-gp and steroid receptors is also recommended.

P-gp efflux can effectively lower the intracellular levels of chemotherapy drugs, in women (who have less P-gp expression), one might therefore expect higher intracellular drug levels and even though the clinical outcome of the same chemotherapy regimens may not be similar between men and women.

DNA topoisomerases are known to be nuclear enzymes that are important targets of topoisomerase inhibitors in cancer chemotherapy. We investigated the protein expression of Topo II α by immunohistochemistry in malignant tissues of advanced gastric cancer. Out of 35 evaluated patients 6 (17.2%) of patients showed positive expression of this marker and this finding is not in agreement with previous report (Coleman *et al*., 2001). Coleman *et al.* (2001) observed Topo II α expression in 16/22 (73%) of their gastric adenocarcinoma cases but this difference may be related to the pathological stages and grades of our cases because all of the evaluated cases were in advanced pathological stage and there was also a significant relationship between Topo II α expression and histological grade ($p = 0.0001$). We observed significant relationship between Topo II α negative immunostaining and gender ($p = 0.0001$) as well as lymphatic invasion ($p= 0.013$) and tumor size ($p = 0.012$). We suggest that the qualitative changes of Topo II α expression could be associated with malignancy grades of tumors. As Yabuki *et al.* (1996) reported Topo II α immunostaining can detected the proliferating cells in routinely processed tissue sections and the altered Topo II α expression in human cancers, may be related to the sensitivity to Topo II α targeted chemotherapeutic agents. These results indicated that the tumor specific chemotherapy with Topo II α inhibitors would be less effective in advanced tumors than those early tumors, because of the lack of expression of Topo II α as their important target. We suggest that the chemotherapy targeting Topo II α enzymes might be less effective in advanced cases when compared with early cases. The present study recognized a significant immunophenotype in advanced gastric carcinoma cases. Although the most prevalent immunophenotype was $P\text{-}gp^{-}$ /Topo II^{-} (48.57%) but we didn't observe its association with clinicopathological features.

Finally considering the key role of positive P-gp or negative Topo II α expression in MDR phenomenon, it could be concluded that groups of patients with P-gp over expression (42.9%) or lack of Topo II α expression (82.8%) would less likely benefit from available chemotherapeutic regimens. Additionally clinicopathological features of patients affect the expression of markers and therefore clinical outcome. Therefore, it is highly recommended to determine the status of tumor markers such as P-gp and Topo II, before deciding about effective chemotherapeutic regimen for patients with gastric cancer.

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